Comparison of volatile compounds produced by wild Lactococcus lactis in miniature Chihuahua-type cheeses

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ORIGINAL PAPER

Comparison of volatile compounds produced by wild *Lactococcus lactis* in miniature Chihuahua-type cheeses

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Abstract Strains of *Lactococcus lactis* used for a long time as starter cultures in the production of cheese have not only acquired special features like fast utilization of lactose, but it is also believed that they have lost certain metabolic capabilities. Certain wild strains of *L. lactis* isolated from vegetables or raw milk products are able to generate flavors different from those produced by industrial strains. The aim of this work was to assess the production of volatile compounds in miniature Chihuahua-type cheeses manufactured with different strains of *L. lactis* isolated from vegetables, raw milk products, and industrial cultures. There was variation among volatile profiles in the miniature cheeses manufactured with different strains of *L. lactis*. However, some compounds were seen in most of the cheeses such as acetic, lactic, butyric, and caproic acids, acetoin, 3-methyl-1-butanol, and 2,3-butanediol. The source of isolation of the strains (plants, raw milk products, and industrial cultures) did not have a clear influence on the production of volatile compounds in miniature cheeses. According to principal component analysis, 19 out of 21 strains of *L. lactis* produced volatile profiles similar to the three pasteurized Chihuahua cheeses analyzed, but only 2 strains generated profiles similar to the commercial raw milk cheese. However, further research is required to understand the metabolic and genetic differences of these strains.

Keywords *Lactococcus lactis* · Wild strains · Volatile compounds · Chihuahua cheese

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1 Introduction

Chihuahua cheese or Mennonite cheese is one of the most popular cheeses in Mexico and the Hispanic community in the USA (Tunick et al. 2008). The color of Chihuahua cheese is yellow and turns golden yellow with age. Its consistency is sliceable, semi-hard, and its sensory characteristics are similar to 1 month-aged Cheddar; although rheological analysis suggests it is more like fresh Colby cheese (Gutiérrez-Méndez and Nevárez-Moorillon 2009). Artisanal Chihuahua cheese is manufactured with unpasteurized milk; nevertheless, nowadays, it is also produced at a large scale using pasteurized milk. According to Van Hekken et al. (2006), Chihuahua cheeses manufactured with unpasteurized milk have a stronger flavor and softer texture than Chihuahua cheeses made with pasteurized milk.

Flavors are chemical sensations produced by certain molecules released from the food during eating (Voilley and Etiévant 2006). The pleasant flavors perceived by consumers in fermented dairy products come from the correct balance of flavor compounds (Smit et al. 2005). Lactic acid bacteria (LAB) provide a high contribution of enzymes that transform the milk components into flavor compounds (Santos et al. 2003). *Lactococcus lactis* has been involved in cheese making since ancient times, and nowadays, these bacteria are most widely used as a starter culture in the manufacture of cheese (Klijn et al. 1995). The natural niches of *L. lactis* are plant environments such as grass, corn leaves, sweet peas, cucumbers, and beans. This microorganism has adapted to other environments including soil, cow's skin (mainly udder and teats), and milk (Desmasures et al. 1997; Doman-Pytka et al. 2004; Nomura et al. 2006). The adaptation of *L. lactis* to milk or milk-related environments has promoted the acquisition of special features like fast lactose utilization (Mills et al. 2006; Gutiérrez-Méndez et al. 2010). However, the adaptation of these bacteria to rich nutrient environments like milk also has caused the loss of certain metabolic capabilities. For example, plant-derived *Lactococci* have a more efficient use of peptides and greater tolerance to stress than milk-derived strains of *L. lactis* (Nomura et al. 2006; Picon et al. 2010).

Some authors have proposed that wild strains of *L. lactis* isolated from vegetables or raw milk products are able to generate flavors different from those produced by industrial strains (Ayad et al. 1999). Certain flavor characteristics observed in artisanal raw milk cheeses are poorly detected in cheeses manufactured with pasteurized milk (Centeno et al. 2002). Therefore, the use of wild strains of *L. lactis* for the development of new flavors or flavors that resemble those perceived in raw milk cheeses may have potential. The aim of this work was to assess the production of volatile compounds in miniature Chihuahua-type cheeses manufactured with different strains of *L. lactis* isolated from vegetables, commercial starter cultures, and raw milk products. Volatile compounds observed in miniature cheeses were also compared with those found in commercial raw and pasteurized milk Chihuahua cheeses.

2 Materials and methods

2.1 Microorganisms

The 21 strains of *L. lactis* used in this study (except the ATCC strain) were obtained and partially characterized in a previous work (Gutiérrez-Méndez et al. 2008b). These strains were isolated from raw milk dairy products, vegetables, and different commercial dairy starter cultures (Table 1). All the strains were kept at −20 °C in an M17 broth (Difco laboratories, Detroit Mich.) with 40% (*v*/*v*) glycerol before use. Additionally, the citrate-fermenting capacity of the strains was assessed using the Kempler and McKay (KMK) agar (Kempler and McKay 1980). The production of acetoin by the strains was detected by the Voges-Proskauer (VP) reaction (Passerini et al. 2013).

2.2 Chihuahua cheese samples

Five different brands of Chihuahua cheese were bought from the local market and stored at 4 °C. The cheese brands were chosen based on a previous survey that determined which were the top selling brands in Chihuahua city (Almanza-Rubio et al. 2013). Mennonite cheese factories located in Cuauhtémoc (Chihuahua state, Mexico) produced all the cheese brands used in this study. The brands used were as follows: Queso Menonita (brand 1), Clavel (brand 2), Los Pinos (brand 3), Lacmeno (brand 4), and Laurel (brand 5). Only one brand (brand 1) was a cheese manufactured with raw milk.

2.3 Manufacture of miniature Chihuahua-type cheeses

Miniature model cheeses were produced in the laboratory under controlled conditions in order to minimize the manufacture variations and reduce the microbial contamination. The protocol used was previously developed in the laboratory, to reproduce similar characteristics than commercial Chihuahua cheeses (Gutiérrez-Méndez et al. 2013). Each strain of *L. lactis* was grown individually in sterile milk (3% fat) at 37 °C over 18 h. These fermented milks were used as inoculum in the production of miniature

Table 1 Strains of *Lactococcus lactis* used in the manufacture of Chihuahua-type miniature cheeses

Strain	Source of isolation	Strain	Source of isolation
11454	ATCC (American Type Culture Collection)	EZ01cb-2	LSC (EZAL, Rhodia Food, France)
C272	Raw milk Chihuahua cheese[a]	EZ02a	LSC (EZAL, Rhodia Food, France)
DE01b	Raw milk Chihuahua cheese[b]	EZ03b	LSC (EZAL, Rhodia Food, France)
Rq07	Whey cheese	FDVSBS10	LSC (Chr Hansen, Denmark)
Alf-2	Lucerne (*Medicago sativo*)	KK01	LSC (Danisco, Denmark)
BB07	Beetroot (*Beta vulgaris*)	KK05	LSC (Danisco, Denmark)
EJ06	Green beans (*Phaseolus vulgaris*)	Li	LSC (Chr Hansen, Denmark)
MH05	Corn leaves (*Zea maiz*)	MA16	LSC (Chr Hansen, Denmark)
MM11	Corn earn (*Zea miz*)	MM101	LSC (Danisco, Denmark)
CM01	LSC (EZAL, Rhodia Food, France)	Pk04	LSC (Danisco, Denmark)
EZ01ab	LSC (EZAL, Rhodia Food, France)		

Vegetable samples were obtained from local producers in Chihuahua State, Mexico

LSC lyophilized starter culture of mesophilic lactococci

[a] Cheese manufactured in Cuauhtémoc, Chihuahua, Mexico

[b] Cheese manufactured in Delicias, Chihuahua, Mexico

Chihuahua-type cheese. The milk used for the manufacture of cheeses was obtained from the Faculty of Animal Science and Ecology (University Autonomous of Chihuahua, Chihuahua, Mexico). The milk was standardized to obtain the following composition: 32.3±2.3 g.L^{-1} fat, 28.1±0.2 g.L^{-1} protein, and 40.0±0.6 g.L^{-1} lactose. Portions of 250 g of pasteurized milk were poured into four 500-mL glass beakers. Milk portions were added 1% (*v*/*v*) with the inoculum of the corresponding strain of *L. lactis*. The milk was incubated 1 h at 32 °C and 100 μL of $CaCl_2$ 6.6 mol.L^{-1} and 13.75 μL of chymosin (Chy-Max, Chr Hansen, Horsholm, Denmark) added. After 1 h of incubation at 32 °C, the coagulum was cut with a stainless steel spatula in small cubes of 0.8 cm^3, held for 10 min, and then stirred at 150 rpm for 60 min on an orbital shaker. The whey was drained and the curd warmed to 38 °C in a water bath (IsoTemp 210, Fisher Sci, Iowa, USA) until pH reached 5.6. Then, the curd was salted at 1% (*w*/*w*) NaCl and transferred into metallic u-bottom tubes and centrifuged at 1,700×*g* for 30 min at room temperature. The expelled whey was drained, and the curd was centrifuged again for 60 min at 1,700×*g*. Mini cheeses were removed from the tubes and dipped into a sorbate (0.3%*w*/*v*) and natamycin (0.002%*w*/*v*) solution. Cheeses were stored unpacked at 4 °C and 75% of relative humidity for 1 week in order to remove the excess of moisture in the cheeses. Finally, mini cheeses were wiped with tissue paper, packed in sealed polyethylene bags (16.5 cm×14.9 cm), and stored at 10 °C for 1 month. Miniature Chihuahua cheeses had an average size of 4 cm (diameter)×1.8 cm (height) and a weight of 22±0.98 g. Three mini cheeses were manufactured with each separate strains of *L. lactis*.

2.4 Compositional analysis of cheeses

Cheeses were analyzed for moisture, protein, fat, and ash content using AOAC methods: 926.08, 991.22, 905.02, and 935.42, respectively (AOAC 1998). The pH of the cheeses was measured with a pH meter (Pinnacle, Corning, NY, USA) according to the AOAC method 981.12 (AOAC 1998).

2.5 Analysis of volatile compounds

The protocol used for the analysis of volatile compounds in cheese was adapted from Bourdat-Deschamps et al. (2004). Volatile compounds in miniature and commercial cheeses were extracted by solid phase microextraction (SPME) using a fiber of 1 cm in length coated with a film of (75 μm) Carboxen/polydimethylsiloxane (Supelco, Bellefonte, PA, USA) and analyzed by gas chromatography–mass spectrometry (GC-MS). The cheese samples were prepared as follows: 20 g of grated cheese and 10 g of NaCl were added to a 250-mL container (Nalgene Nunc International, NY, USA). Then, the CAR/PDMS fiber was placed into the container headspace and held for 1 h at 45 °C. The fiber was retracted and injected into the chromatograph inlet and desorbed by 31 min at 200 °C. Volatile compounds were separated on a GC-MS equipment (Autosystem XL, Perkin-Elmer, Norwalk, CT, USA) with a polyethylene glycol (Elite-Wax ETR, Perkin-Elmer, Norwalk, CT, USA) column (30 m×0.25 mm i.d.) using the following conditions: initial temperature 45 °C held for 3 min, heating to 150 °C at 5 °C.min^{-1} and held for 1 min, heating to 200 °C at 10 °C.min^{-1}, and holding for 1 min. Ionization was performed in the mass spectrometer (Turbo mass gold, Perkin-Elmer, Norwalk, CT, USA) applying an electronic impact of 70 eV. Autotune calibration was routinely performed before each injection sequence. Compounds were presumptively identified by NIST 02 Mass Spectral Database version 2.0 and their mass spectra compared to those reported in the literature.

2.6 Statistical analysis

A multivariate analysis was performed using the relative peak areas of total ion chromatograms. Principal component analysis (PCA) was calculated using a covariance matrix of standardized data collected from peak areas. Factor scores from PCA were used as Euclidean distance matrix for non-hierarchical (k-means) cluster analysis. Statistical analysis was carried out with the software Minitab 16 (Minitab Inc, State College, PA, USA).

3 Results

3.1 Fermentation of citrate and production of diacetyl

Most of the strains of *L. lactis* used in this study were positive to citrate fermentation using the KMK agar (KMK^+) method, and only the strains Li and MA16 were considered as citrate negative (KMK^-). According to the Voges-Proskeaur (VP) reaction, the majority of the strains were able to produce acetoin during growth on milk (VP^+), except strains BB07, MM11, CM01, and MA16 (VP^-).

3.2 Characterization of miniature and commercial Chihuahua cheeses

The compositional parameters of miniature Chihuahua cheeses and commercial Chihuahua cheeses were similar (*t* Student, $P<0.05$). The average composition of miniature cheeses (expressed as g.100 g^{-1}) was as follows: moisture 40.6±3.2, protein 23.2±2.3, fat 28.0±1.8, ash 2.6±0.7, and pH 5.3±0.2. The average composition of commercial Chihuahua cheeses (expressed as g.100 g^{-1}) was as follows: moisture 41.1±2.9, protein 23.5±1.8, fat 27.7±2.1, ash 3.0±0.4, and pH 5.7±0.2.

3.3 Volatile compounds in Chihuahua cheese brands

In this work, four of the brands analyzed were cheeses manufactured with pasteurized milk (brands 2, 3, 4, and 5), and only one brand (brand 1) was a cheese made with raw milk. The cheeses manufactured with pasteurized milk require the addition of commercial starter cultures, whereas raw milk uses the natural flora of the milk to ferment the curd. As was expected, the type and proportion of volatile compounds varied among Chihuahua cheese brands (Fig. 1). However, some volatile compounds were found in all cheese brands, like certain organic acids (acetic, formic, butyric, caproic, and lactic acid) as well as 2,3-butanediol (Tables 2 and 4). The presence of formic acid in all cheese brands was unexpected, because this compound has a pungent, penetrating odor and has only been identified in some cheeses like Cheddar cheese (Mullin and Emmons 1997). All the cheeses manufactured with pasteurized milk showed some

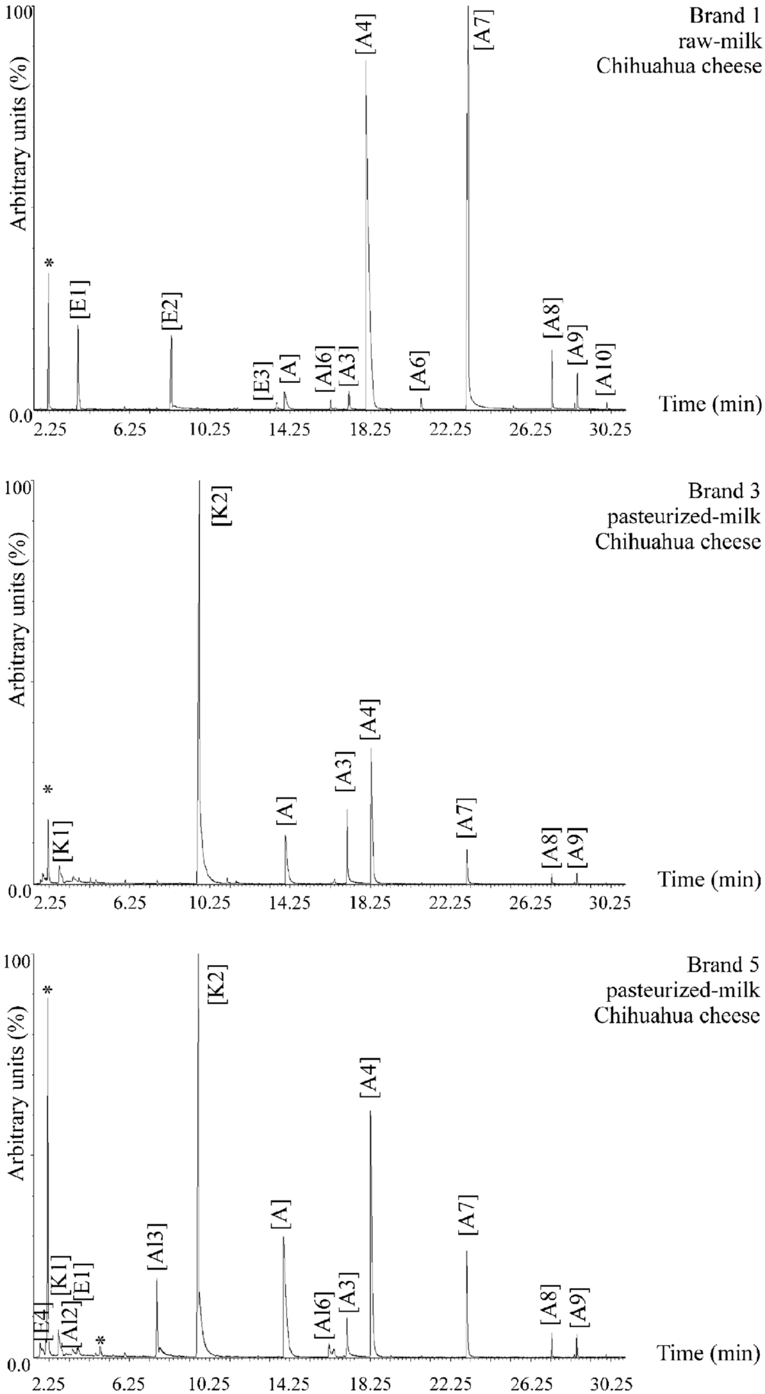

Fig. 1 Volatile compound profiles observed in pasteurized milk Chihuahua cheese (brands 5 and 3) and raw milk Chihuahua cheese (brand 1) by gas chromatography–mass spectrometry (GC-MS) and solid phase micro extraction (SPME). Details of volatile compounds are shown in Table 2

Table 2 List of major volatile compounds identified in miniature Chihuahua-type cheeses and five of the best selling brands of Chihuahua cheese

Id	Compound	Retention time[a] (min)
	Acids	
A	Methanoic acid (formic)	13.6–14.2
A1	Ethanoic acid (acetic acid)	13.8–14.3
A2	2-Methylpropanoic acid (isobutyric acid)	16.9–17.0
A3	2-Hydroxypropanoic acid (lactic acid)	17.1–17.3
A4	Butanoic acid (butyric acid)	18.0–18.4
A5	3-Methylbutanoic acid (isovaleric acid)	19.3–19.4
A6	Pentanoic acid (valeric acid)	20.8
A7	Hexanoic acid (caproic acid)	23.1–23.5
A8	Octanoic acid (caprylic acid)	27.3–27.4
A9	2-4-Hexadienoic acid (sorbic acid)	28.4–29.7
A10	Decanoic acid	30.0–30.1
	Alcohols	
Al2	2-Butanol	3.4
Al3	3-Methyl,1-butanol	7.5–7.8
Al4	2-Methyl-2,4-pentanediol	11.1
Al5	4,5-Octanediol,2,7-dimethyl	14.4
Al6	2,3-Butanediol	16.2–16.5
	Aldehyde	
Ah1	3-Methylpropanal	2.0
	Esters	
E1	Ethyl butyrate	3.7
E2	Ethylhexanoate	8.3
E3	Ethyl octanoate	13.6
E4	1,3-Propanediol,diacetate	1.9
	Ketones	
K1	3-Methyl-2-butanone	2.7–2.9
K2	3-Hydroxybutanone (acetoin)	9.4–9.9
K3	4-Hydroxy-4-methyl-2pentanone	11.6–11.7
	Terpene	
T1	Limonene	7.1–7.2

[a] Range of time obtained from all the cheeses analyzed (21 cheese treatments and 5 commercial brands of Chihuahua cheese)

volatile compounds like 3-methyl-2-butanone and acetoin, but these compounds were not found in the cheese manufactured with raw milk. Likewise, some volatile compounds observed in the raw milk cheese were not found in pasteurized milk cheeses: valeric acid, decanoic acid, ethylhexanoate, and 1,3-propanediol-diacetate. Pasteurized milk cheeses had acetoin and butyric acid as the major volatile components, whereas the raw milk cheese had caproic and butyric acids as the main volatile components (Fig. 1).

3.4 Volatile compounds in miniature Chihuahua-type cheeses

The miniature cheeses manufactured with different strains of *L. lactis* had a wide variation in their volatile profiles (Tables 3 and 4). Nevertheless, all the miniature cheeses had certain volatile compounds like acetic, lactic, butyric, and caproic acids, as well as acetoin, 3-methyl-1-butanol (except strain MM11), and 2,3-butanediol (except the ATCC strain). These acids, alcohols, and ketone are compounds commonly found in cheese (Barbieri et al. 1994; Garde et al. 2002; Bertolino et al. 2011; Hou et al. 2014).

According to the principal component analysis and the k-mean clustering analysis (Fig. 3), the cheeses were organized in three clusters. The first cluster included six cheeses manufactured with strains isolated from commercial cultures (FDVSBS, MA16, KK05, EZ01cb-2, KK01, EZ02a), two cheeses manufactured with strains isolated from raw milk cheeses (De01b, C272), and three cheeses manufactured with strains isolated from vegetables (Alf-2, BB07, EJ06). The volatile profiles of the cheeses that belong to this cluster were similar to the volatile profiles of two commercial Chihuahua cheeses (brands 2 and 3). The main characteristic of these cheeses was the high proportion of ketones (acetoin, 3-methyl-2-butanone, and 4-hydroxy-4-methyl-2pentanone) in comparison with the other volatile compounds (i.e., strain FDVSBS; Fig. 2). Additionally, isobutyric acid was identified in some cheeses of the first cluster like those made with the strains C272 and De01b. The second cluster included the volatile profiles of five miniature cheeses made with strains isolated from commercial cultures (EZ01ab, MM101, Li, EZ03b, PK04), one cheese manufactured with a strain isolated from corn earn (MM11), one cheese manufactured with a strain isolated from whey cheese (Rq07), and one commercial pasteurized milk cheese (brand 5). Some of the cheeses of this cluster showed flavor compounds like limonene (strains EZ03b, MM101, and MM11) or decanoic acid (strain MM11). The volatile compounds most abundant in the cheeses of the second cluster were as follow: acetic acid, lactic acid, butyric acid, ethylbutyrate, and 3-methyl-1-butanol. In contrast to the first cluster, these compounds were found in higher proportion than acetoin (i.e., strain Rq07; Fig. 2). The third cluster comprised the volatile profiles of one miniature cheese manufactured with a strain isolated from commercial culture (CM01), one miniature cheese manufactured with a strain isolated from corn leaves (MH05), and two commercial cheeses (brands 1 and 4). The patterns of volatile compounds that presented these cheeses were very different to the volatile profiles seen in the other mini cheeses and those observed in three out five commercial Chihuahua cheeses. The most abundant volatile compounds in the cheeses of the third cluster were as follows: lactic acid, butyric acid, caproic acid, ethylbutyrate, and 2,3-butanediol (i.e., strain MH05; Fig. 2). Some volatile compounds like valeric acid, ethyl ethanoate, and ethyl octanoate were found exclusively in the raw milk cheese (brand 1) belonging to this third cluster. This commercial Chihuahua cheese made with raw milk (brand 1) had also large differences with most of the commercial pasteurized milk cheeses (brands 2, 3, and 5), whereas there were some similarities with brand 4. Furthermore, only the two mini cheeses made using *L. lactis* strains CM01 and MH05 had volatile profiles similar to the raw milk cheese.

Table 3 Volatile compounds (acids and terpene) observed in miniature Chihuahua-type cheeses manufactured with diverse strains of *L. lactis* and commercial Chihuahua cheeses made from raw and pasteurized milk

	Source	Cheese sample	Volatile compounds											
			A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	T1
			Relative peak areas expressed in arbitrary units (*E* + 06)											
First cluster	P-M	Brand 2	3.07	–	–	2.09	5.8	0.29	–	0.29	–	–	–	–
	C	FDVSBS	–	6.21	–	–	10.6	–	–	2.79	–	3.28	–	–
		ATCC	–	6.11	–	0.6	1.03	–	–	0.46	–	0.4	–	–
	C	MA16	–	1.89	–	0.42	3.82	0.47	–	1.17	0.23	0.55	–	–
	V	EJ06	–	8.4	–	2.11	13.9	1.32	–	2.43	–	1.17	–	1.56
	R	C272	–	3.34	4.21	–	7.51	0.88	–	2.64	0.34	2.41	–	–
	R	De01b	–	1.55	0.53	3.4	7.85	2.58	–	1.49	0.28	0.7	–	–
	C	KK05	–	2.96	–	9.08	22.8	1.38	–	4.87	0.4	1.46	–	–
	V	Alf-2	–	21.9	–	16.5	10.2	0.75	–	3.09	–	1.79	–	–
	C	KK01	–	7.55	–	6.12	1.8	–	–	2.57	0.27	0.73	–	–
	P-M	Brand 3	2.11	–	–	1.45	4.11	–	–	0.67	0.13	0.13	–	–
	C	EZ02a	–	14.6	–	17.9	18.3	2.15	–	3.39	–	2.3	–	–
	V	BB07	–	5.08	–	12.4	3.89	–	–	1.71	–	1.1	–	–
	C	EZ01cb-2	–	10.9	–	10.7	6.03	–	–	1.75	–	2	–	–
Second cluster	C	Pk04	–	11.5	–	7.31	14.7	1	–	5.06	0.42	2.06	–	–
	C	EZ03b	–	17.2	–	35.3	23.4	5.57	–	5.04	0.3	0.86	–	1.44
	C	Li	–	2.25	–	1.59	7.05	0.9	–	1.98	–	1.2	–	–
	C	MM101	–	13.6	–	47.9	18.3	2.83	–	3.91	–	1.06	–	0.54
	P-M	Brand 5	4.98	–	–	0.65	5.56	–	–	1.5	0.22	0.2	–	–
	V	MM11	–	456	–	216	129	3.9	–	18.8	–	–	12.4	54.9

Table 3 (continued)

	Source	Cheese sample	Volatile compounds											
			A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	T1
			Relative peak areas expressed in arbitrary units ($E + 06$)											
	C	EZ01ab	–	40.6	–	14.3	29.2	2.58	–	6.46	–	–	–	–
	R	Rq07	–	4.63	–	2.64	3.71	–	–	3.78	0.46	1.21	–	–
Third cluster	P-M	Brand 4	2.46	–	–	1.42	15	–	–	2.75	0.38	0.48	–	–
	V	MH05	–	11.4	–	36.5	11.8	1.09	–	4.58	0.34	1.31	–	–
	C	CM01	–	27.6	–	70.3	10.5	0.74	–	3.35	–	1.56	–	–
	R-M	Brand 1	4.36	–	–	1.97	96.6	–	1.12	62.9	4.17	0.37	0.37	–

n=3; clusters determined by principal component analysis; Id of volatile compounds are shown in Table 2

V strain isolated from vegetable, *C* strain isolated from industrial starter culture, *R* strain isolated from raw milk product, *P-M* pasteurized milk Chihuahua cheese, *R-M* raw milk Chihuahua cheese

Table 4 Volatile compounds (alcohols, aldehydes, esters, and ketones) observed in miniature Chihuahua-type cheeses manufactured with diverse strains of *L. lactis* and commercial Chihuahua cheeses made from raw and pasteurized milk

Source		Cheese sample	Volatile compounds												
			Al2	Al3	Al4	Al5	Al6	Ah1	E1	E2	E3	E4	K1	K2	K3
			Relative peak areas expressed in arbitrary units (*E* + 06)												
First cluster	P-M	Brand 2	–	0.67	0.48	–	1.17	–	–	–	–	–	4.53	736	–
	C	FDVSBS	–	17.2	–	–	4.4	–	–	–	–	–	–	289	6.17
		ATCC	–	0.48	–	–	–	–	–	–	–	–	1.72	74.6	0.89
	C	MA16	–	0.29	–	–	3.68	–	–	–	–	–	2.42	71.9	–
	V	EJ06	–	1.64	0.75	–	18.7	–	–	–	–	–	9.21	209	–
	R	C272	–	0.47	0.3	–	11.7	–	–	–	–	–	2.76	88.2	–
	R	De01b	–	1.85	0.28	–	4.69	–	–	–	–	–	2.18	56.1	–
	C	KK05	–	2.49	0.58	–	5.44	–	0.73	–	–	–	7.61	131	–
	V	Alf-2	–	11.4	–	–	6.25	–	–	–	–	–	–	155	–
	C	KK01	–	22.4	–	2.88	5.57	–	–	–	–	–	1.64	48.6	1.38
	P-M	Brand 3	0.29	–	0.07	–	0.13	–	0.11	–	–	0.17	0.76	13.7	–
	C	EZ02a	–	15.5	0.9	0.7	5.61	–	0.97	–	–	–	3.28	93.1	–
	V	BB07	–	40.7	–	1.96	12.8	–	–	–	–	–	2.12	59	1.13
	C	EZ01cb-2	–	61.2	–	3.55	5.92	0.38	–	–	–	–	2.97	79	28.6
Second cluster	C	Pk04	–	6.18	0.37	–	1.97	0.59	0.35	–	–	–	2.29	33.3	–
	C	EZ03b	–	7.08	–	–	31.6	–	0.74	–	–	–	5.14	88.3	–
	C	Li	–	1.07	–	–	7.17	–	–	–	–	–	3.43	11.6	–
	C	MM101	–	10.4	0.32	–	25.4	–	–	–	–	–	–	68.4	–
	P-M	Brand 5	0.14	1.41	–	–	0.33	–	0.2	–	–	0.17	0.72	7.94	–
	V	MM11	–	–	–	–	131	–	5.71	–	–	–	–	377	–

Table 4 (continued)

Source		Cheese sample	Volatile compounds												
			Al2	Al3	Al4	Al5	Al6	Ah1	E1	E2	E3	E4	K1	K2	K3
			Relative peak areas expressed in arbitrary units ($E + 06$)												
	C	EZ01ab	–	98.0	–	7.78	7.43	–	–	–	–	–	3.8	54.2	–
	R	Rq07	–	68.4	–	2.2	1.63	–	1.1	–	–	–	–	25.9	1.22
Third cluster	P-M	Brand 4	–	0.17	0.11	–	2.07	0.07	–	–	–	0.16	5.11	7.07	–
	V	MH05	–	8.62	–	–	24.8	–	0.31	–	–	–	0.79	17.6	1.25
	C	CM01	–	11.7	–	–	36.6	11.4	0.86	–	–	–	–	11.7	–
	R-M	Brand 1	–	–	–	–	0.84	–	8.54	7.46	0.51	–	–	–	–

n=3; clusters determined by principal component analysis; Id of volatile compounds are shown in Table 2

V strain isolated from vegetable, *C* strain isolated from industrial starter culture, *R* strain isolated from raw milk product, *P-M* pasteurized milk Chihuahua cheese, *R-M* raw-milk Chihuahua cheese

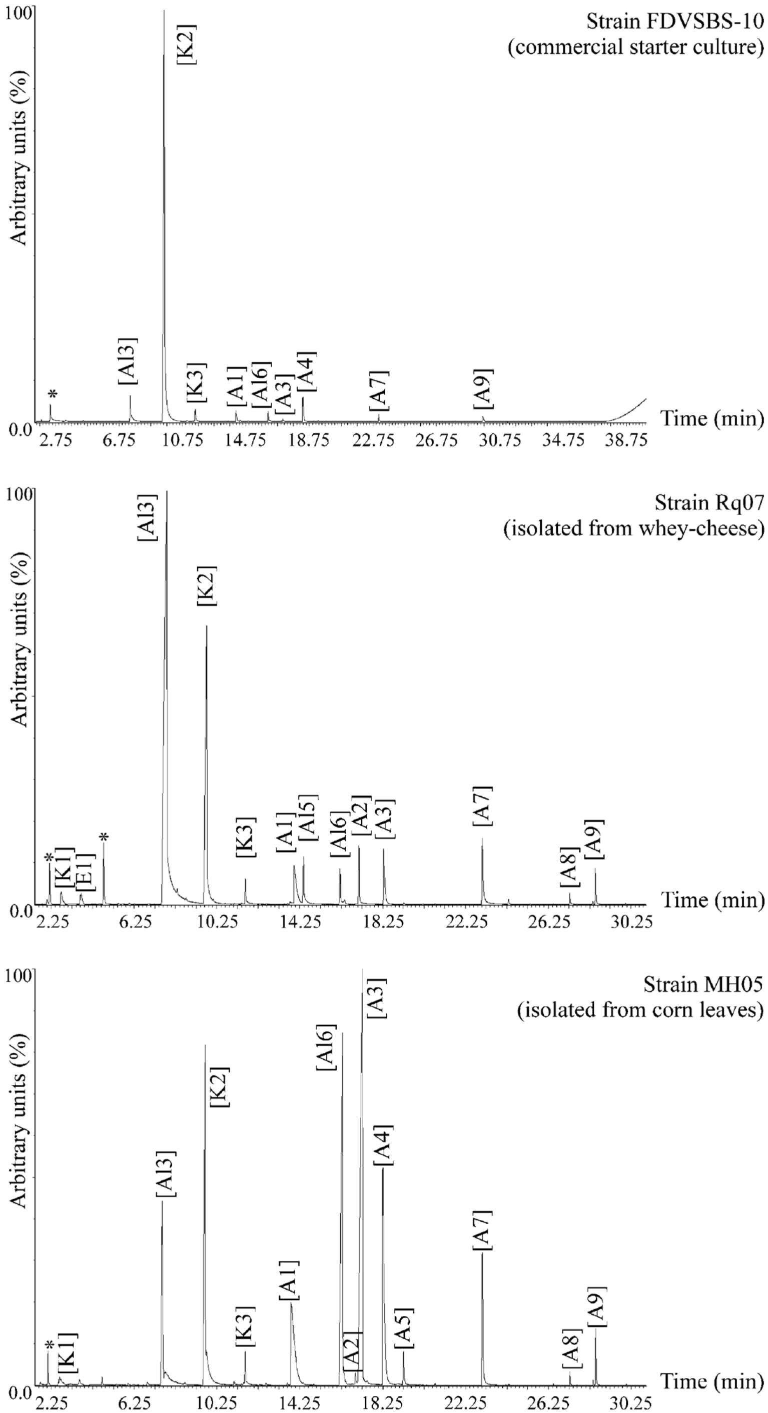

Fig. 2 Volatile compound profiles obtained by gas chromatography–mass spectrometry (GC-MS) and solid phase micro extraction (SPME) in miniature Chihuahua-type cheeses manufactured with three different strains of *L. lactis* isolated from a commercial starter culture (FDVSBS-10), whey cheese (Rq07), and a vegetable sample (MH05). Details of volatile compounds are shown in Table 2

4 Discussion

There is the perception by local consumers that raw milk Chihuahua cheeses have stronger and more pleasant flavor than most of the pasteurized milk cheeses. Van Hekken et al. (2006) reported that raw milk Chihuahua cheeses had more intense sour and bitter flavor than pasteurized milk cheeses. It was anticipated that some differences among the volatile profiles of cheeses manufactured with pasteurized milk and raw milk cheeses will occur. These differences can be attributed to many different factors but mainly to the heat treatment of the cheese milk and the variety of microorganisms present in the milk and the cheeses. Bricker et al. (2005) reported that Chihuahua cheeses manufactured with pasteurized milk contained mostly LAB (mesophilic lactococci, *Lecuconostoc* spp., thermophilic lactococci and lactobacilli), whereas raw milk cheeses had LAB, coliforms, enterococci, and coagulase-positive staphylococci. Nevertheless, it is difficult to establish the effect of microorganisms other than LAB on the production of volatile compounds. On the other hand, it is widely known that heat treatment of the milk increases the presence of certain volatile compounds (fatty acids, esters, ketones, and aldehydes) modifying the flavor in the milk (Hettinga et al. 2008). According to Zabbia et al. (2012), cooked and flat flavors that develop in the milk during thermal processing derive essentially from Maillard compounds including methyl ketones, furans, pyrazines, sulfur, and nitrogen-containing compounds. This could explain the absence of certain ketones (3-methyl-2-butanone and acetoin) in the cheeses manufactured with raw milk (Fig. 1).

The miniature cheeses (1 month-aged) made with different strains of *L. lactis* showed a wide variation in their volatile profiles. These differences in volatile profiles were generally not related with the source of isolation of the strains (Tables 3 and 4; Fig. 3). Nevertheless, one strain isolated from artisanal whey cheese (strain Rq07) and one strain isolated from corn leaves (strain MH05) had unusual patterns of volatile compounds (Figs. 2 and 3). In a previous study, Gutiérrez-Méndez et al. (2008b) assessed the aroma of milks fermented with different strains of *L. lactis* isolated from vegetables, raw milk cheeses, and industrial starter cultures. The analysis of these fermented milks using an electronic nose did not show a clear relationship between the source of isolation and the ability of lactococci to produce aroma, though some milks fermented with wild strains presented stronger yogurt-like aroma than those milks fermented with industrial strains. Ayad et al. (1999) reported that milks fermented with some wild strains of *L. lactis* isolated from goat and cow milk contained higher levels of certain volatile compounds than milks fermented with industrial strains. Nomura et al. (2006) reported that milks fermented with plant-derived strains of *L. lactis* had a similar flavor than milks fermented with reference strains; however, the milk fermented with some strains of *L. lactis* isolated from cow milk had a malty taste. Centeno et al. (2002) reported that ewes' cheese manufactured with industrial strains of *L. lactis* and wild strains of *L. lactis* isolated from raw milk cheeses had higher abundance of volatile compounds that cheese manufactured only with industrial strains. From results obtained by different authors (Ayad et al. 1999; Centeno et al. 2002; Nomura et al. 2006; Gutiérrez-Méndez et al. 2008b), it could be established that not all the wild strains of *L. lactis* have the capability to generate more volatile compounds than industrial strains, but some particular wild strains of *L. lactis* can produce unusual compounds and/or higher abundance of volatiles.

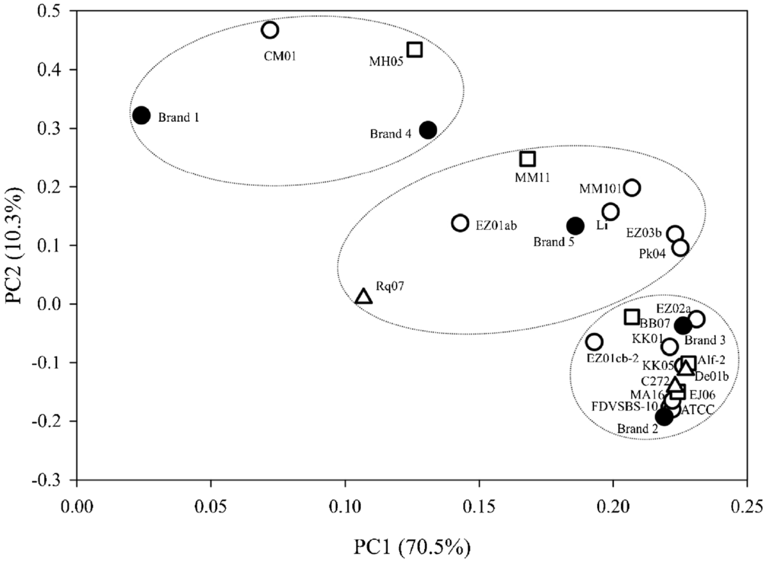

Fig. 3 Principal component analysis (PCA) of volatile compounds observed in five brands of Chihuahua cheese and miniature Chihuahua-type cheeses (1-month-old) manufactured with different strains of *L. lactis*: *white circles* strains isolated from commercial cultures, *white squares* strains isolated from vegetables, *white triangles* strains isolated from raw milk Chihuahua cheeses, *black circles* commercial Chihuahua cheeses. Clusters were defined by k-mean clustering analysis

L. lactis produces different flavor compounds during its growth in the milk, curd, and cheese mostly from the fermentation of citrate and protein degradation. The citrate fermentation generates important flavor compounds like acetaldehyde, diacetyl, acetoin, and 2,3-butanediol (Goupry et al. 2000). All the miniature cheeses manufactured with different strains of *L. lactis* contained acetoin and 2,3-butanediol. Probably because most of the strains used were identified as citrate positive by KMK agar (KMK$^+$) and positive to acetoin production by the VG reaction (VG$^+$). However, the cheeses manufactured with the strains identified as KMK$^-$ and/or VG$^-$ also presented large amounts of acetoin and 2,3-butanediol. The inconsistencies of these results may be attributed to the lack specificity of the KMK and VG methods. According to Passerini et al. (2013), the KMK and VG methods have poor correlation with the real utilization of citrate and the presence of citrate catabolic genes. These authors also confirmed that strains of *L. lactis* that harbored the *citP* gene and the *citM-G* cluster produced a larger number of aroma compounds than those strains without this genetic information.

On the other hand, degradation of milk proteins by *L. lactis* produces a large variety and amount of volatile compounds. The proteolytic system of LAB is composed of proteinases that initially cleave the milk proteins to peptides, and then, these peptides are hydrolyzed to amino acids by intracellular peptidases. Amino acids are catabolized producing a variety of low molecular weight compounds like aldehydes, alcohols, carboxylic acids, esters, and sulfur compounds (Savijoki et al. 2006). The gene *prtP* that encodes the cell envelope-associate proteinase in *L. lactis* is harbored in extrachromosomal DNA acquired by continuous exposure with other microorganisms that grow in the milk (Mills et al. 2006). For this reason, it has been suggested that plant-derived strains have limited proteolytic activity against caseins (Ayad et al. 1999; Picon et al. 2010). In a previous characterization of the strains of *L. lactis* used in this work, the strains isolated from vegetables were found with low proteolytic activities, though the strain MH05 (isolated from corn leaves) showed a very high proteolytic activity (Nájera-Domínguez and Gutiérrez-Méndez 2013). It is worth noting that strain MH05 produced an unusual volatile profile with a large variety of volatile compounds.

The majority of volatile compounds produced by *L. lactis* come from the catabolism of amino acids (Yvon and Rijnen 2001). The limiting step in the catabolism of amino acids is the depletion of α-ketoglutarate (α-KG), which acts as an amino group acceptor during the transamination step. Some strains of *L. lactis* can synthesize the α-KG from glutamate through the glutamate dehydrogenase (GDH) enzyme (van Kranenburg et al. 2002). For this reason, some authors have suggested that GDH activity could be the most important criteria for the selection of flavor-producing *L. lactis* (Tanous et al. 2002) and nonstarter LAB (Kieronczyk et al. 2001). In the present study, the strains MH05, CM01, and RQ07 produced unusual volatile profiles. These strains have been previously identified with GDH activity. Two out of three strains (MH05 and RQ07) with GDH-NADP activities and one strain with GDH-NAD activity (Gutiérrez-Méndez et al. 2008a). However, other strains similarly confirmed with GDH activities and also used in this work did not produce unusual patterns or large abundance of volatile compounds.

5 Conclusions

The volatile profiles of commercial Chihuahua cheeses made from pasteurized milk were clearly different to that made with raw milk. The capacity of *L. lactis* to produce volatile compounds in miniature cheeses was strain-dependent. The source of isolation of the strains did not influence the production of volatile compounds. Most of the wild strains and industrial strains of *L. lactis* produced similar volatile compounds. Nevertheless, the cheeses manufactured with some particular strains (CM01, MH05, and Rq07) showed unusual profiles of volatile compounds. However, further research is required to understand the metabolic and genetic differences of these strains.

All over the world local governments urge cheese makers to pasteurize the milk during the manufacture of cheese and thus reduce the risk of food-borne pathogenic bacteria. From the point of view of cheese makers and consumers, pasteurized milk cheeses have different flavors than raw milk cheeses. For this reason, there is a growing interest in the use of wild strains of *L. lactis* isolated from raw milk products to obtain similar flavors than artisanal cheeses. However, in this work, it was observed that only few wild strains are able to produce unusual flavor compounds.

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References

Almanza-Rubio JL, Orozco-Mena RE, Gutiérrez-Méndez N (2013) Assessing consumer preference toward Chihuahua cheese and Chihuahua-type cheese. Tecnociencia 7:123–131

AOAC (1998) Official methods of analysis, 16 ed. Association of Official Analytical Chemists, Washington D.C. USA

Ayad EHE, Verheul A, de Jong C et al (1999) Flavour forming abilities and amino acid requirements of *Lactococcus lactis* strains isolated from artisanal and non-dairy origin. Int Dairy J 9:725–735

Barbieri G, Bolzoni L, Careri M, Mangia A (1994) Study of the volatile fraction of Parmesan cheese. J Agric Food Chem 42:1170–1176

Bertolino M, Dolci P, Giordano M et al (2011) Evolution of chemico-physical characteristics during manufacture and ripening of Castelmango PDO cheese in wintertime. Food Chem 129:1001–1011

Bourdat-Deschamps M, LeBars D, Yvon M, Chapot-Chartier M-P (2004) Autolysis of *Lactococcus lactis* AM2 stimulates the formation of certain aroma compounds from amino acids in a cheese model. Int Dairy J 14:791–800

Bricker AL, Van Hekken DL, Guerrero VM, Gardea AA (2005) Microflora isolated from Mexican Mennonite-style cheeses. Food Prot Trends 25:637–640

Centeno JA, Tomillo FJ, Fernández-Garcia E et al (2002) Effect of wild strains of *Lactococcus lactis* on the volatile profile and the sensory characteristics of ewes's raw milk cheese. J Dairy Sci 85:3164–3172

Desmasures N, Opportune W, Guéguen M (1997) *Lactococcus* spp., yeasts and *Pseudomonas* spp. on teats and udders of milking cows as potential sources of milk contamination. Int Dairy J 7:643–646. doi:10.1016/S0958-6946(97)00042-3

Doman-Pytka M, Renault P, Bardowski J (2004) Gene-cassette for adaptation of *Lactococcus lactis* to a plant environment. Lait 84:33–37

Garde S, Carbonell M, Fernández-Garcia E et al (2002) Volatile compounds in hispánico cheese manufactured using a mesophilic starter, a thermophilic starter, and bacteriocin-producing *Lactococcus lactis* subsp. *lactis* INIA 415. J Agric Food Chem 50:6752–6757

Goupry SP, Croguennec T, Gentil E, Robins RJ (2000) Metabolic flux in glucose/citrate co-fermentation by lactic acid bacteria as measured by isotopic ratio analysis. FEMS Microbiol Lett 182:207–211. doi:10.1111/j.1574-6968.2000.tb08896.x

Gutiérrez-Méndez N, Nevárez-Moorillon GV (2009) Chihuahua cheese: the history of a Mexican cheese. Carnilac Ind 24:27–34

Gutiérrez-Méndez N, Valenzuela-Soto E, González-Cordova AF, Vallejo-Cordoba B (2008a) α-Ketoglutarate biosynthesis in wild and industrial strains of *Lactococcus lactis*. Lett Appl Microbiol 47:202–207

Gutiérrez-Méndez N, Vallejo-Cordoba B, González-Cordova AF et al (2008b) Evaluation of aroma generation of *Lactococcus lactis* with an electronic nose and sensory analysis. J Dairy Sci 91:49–57

Gutiérrez-Méndez N, Rodríguez-Figueroa JC, González-Cordova AF et al (2010) Phenotypic and genotypic characteristics of *Lactococcus lactis* strains isolated from different ecosystems. Can J Microbiol 56:432–439

Gutiérrez-Méndez N, Trancoso-Reyes N, Leal-Ramos MY (2013) Texture profile analysis of fresh cheese and Chihuahua cheese using miniature cheese models. Tecnociencia 7:65–74

Hettinga KA, van Valenberg HJF, van Hooijdonk ACM (2008) Quality control of raw cows' milk by headspace analysis. Int Dairy J 18:506–513. doi:10.1016/j.idairyj.2007.10.005

Hou J, Hannon JA, McSweeney PL et al (2014) Effect of curd washing on cheese proteolysis, texture, volatile compounds, and sensory grading in full fat Cheddar cheese. Int Dairy J 34:190–198

Kempler GM, McKay LL (1980) Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. Appl Environ Microbiol 39:926–927

Kieronczyk A, Skeie S, Olsen K, Langsrud T (2001) Metabolism of amino acids by resting cells of non-starter lactobacilli in relation to flavour development in cheese. Int Dairy J 11:217–224. doi:10.1016/S0958-6946(01)00051-6

Klijn N, Weerkamp AH, de Vos WM (1995) Detection and characterization of lactose-utilizing *Lactococcus* spp. in natural ecosystems. Appl Environ Microbiol 61:788–792

Mills S, McAuliffe OE, Coffey A et al (2006) Plasmids of lactococci—genetic accessories or genetic necessities? FEMS Microbiol Rev 30:243–273. doi:10.1111/j.1574-6976.2005.00011.x

Mullin WJ, Emmons DB (1997) Determination of organic acids and sugars in cheese, milk and whey by high performance liquid chromatography. Food Res Int 30:147–151. doi:10.1016/S0963-9969(97)00026-4

Nájera-Domínguez C, Gutiérrez-Méndez N (2013) Autolytic and proteolytic properties of strains of *Lactococcus lactis* isolated from different vegetables, raw-milk cheeses and commercial starter cultures. Food Nutr Sci 4:21–26. doi:10.4236/fns.2013.411A004

Nomura M, Kobayashi M, Narita T et al (2006) Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. J Appl Microbiol 101:396–405

Passerini D, Laroute V, Coddeville M et al (2013) New insights into *Lactococcus lactis* diacetyl- and acetoin-producing strains isolated from diverse origins. Int J Food Microbiol 160:329–336

Picon A, García-Casado MA, Nuñez M (2010) Proteolytic activities, peptide utilization and oligopeptide transport systems of wild *Lactococcus lactis* strains. Int Dairy J 20:156–162. doi:10.1016/j.idairyj.2009.10.002

Santos MV, Ma Y, Caplan Z, Barbano DM (2003) Sensory threshold of off-flavors caused by proteolysis and lipolysis in milk. J Dairy Sci 86:1601–1607. doi:10.3168/jds.S0022-0302(03)73745-X

Savijoki K, Ingmer H, Varmanen P (2006) Proteolytic systems of lactic acid bacteria. Appl Microbiol Biotechnol 71:394–406. doi:10.1007/s00253-006-0427-1

Smit G, Smit BA, Engels WJM (2005) Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiol Rev 29:591–610

Tanous C, Kieronczyk A, Helinck S et al (2002) Glutamate dehydrogenase activity: a major criterion for the selection of flavour-producing lactic acid bacteria strains. Antonie Van Leeuwenhoek 82:271–278

Tunick MH, Van Hekken DL, Molina-Corral FJ et al (2008) Queso Chihuahua: manufacturing procedures, composition, protein profiles, and microbiology. Int J Dairy Technol 61:62–69

Van Hekken DL, Drake MA, Molina-Corral FJ et al (2006) Mexican Chihuahua cheese: sensory profiles of young cheese. J Dairy Sci 89:3729–3738

Van Kranenburg R, Kleerebezem M, Vlieg JVH et al (2002) Flavour formation from amino acids by lactic acid bacteria: predictions from genome sequence analysis. Int Dairy J 12:111–121

Voilley A, Etiévant P (2006) Flavour in food. CRC Press, Boca Raton

Yvon M, Rijnen L (2001) Cheese flavour formation by amino acid catabolism. Int Dairy J 11:185–201

Zabbia A, Buys EM, DeKock HL (2012) Undesirable sulphur and carbonyl flavor compounds in UHT milk: a review. Crit Rev Food Sci Nutr 52:21–30